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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Most soy-breast cancer epidemiological studies conclude that Asian women consuming a traditional diet high in soy products have a low incidence of breast cancer. We have demonstrated that prepubertal exposure to genistein, the primary isoflavone of soy, protects against chemically-induced mammary cancer. The purpose of this work was/is to determine if adult exposure to genistein will protect against chemically-induced mammary cancer and to investigate DNA methylation of estrogen receptor genes as the molecular mechanism of genistein chemoprevention. To date, we have determined that adult only exposure to genistein does not protect against dimethylbenz(a)anthracene-induced mammary cancer. However, prepubertal plus adult exposure to 250 mg genistein/kg AIN-76A diet protected against DMBA-induced mammary cancer. Preliminary data shows that genistein increases DNA methyltransferase activity, suggesting that exposure to genistein prepubertally may imprint molecular events in the mammary gland that determines the "blue print" from which the mammary cells responds to future hormonal and/or xenobiotic response. In the third year, we will investigate DNA methylation of estrogen receptor-alpha gene as the molecular mechanism for genistein imprinting against mammary cancer.

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INTRODUCTION

We have previously demonstrated that short-term exposure of rats to genistein, a soy phytoestrogen, early in postnatal life suppressed chemically-induced mammary cancer in adult rats (1, 2). This novel finding supports the epidemiological reports that Asian women consuming a traditional diet high in soy products have a low incidence of breast cancer (3-6). Furthermore, adjustment for migration rates of Asians to the U.S. revealed that the second, but not the first, generation lose this protection (6, 7). This suggests that exposure to soy early in life confers life-time protection against breast cancer. Since short-term genistein treatment early in postnatal life exerted long-term protection against chemically-induced mammary cancer, we have hypothesized that genistein caused this effect via an imprinting mechanism. For this research, we proposed to investigate 1) the potential of adult genistein treatment to alter susceptibility for breast cancer and 2) DNA methylation of estrogen receptor genes as the molecular mechanism of action.

BODY

Specific Aim 1) To determine the risk of mammary cancer from adult exposure to genistein. This was investigated in the rat-DMBA mammary cancer model.

In the first experiment, we have investigated 2 groups of rats fed AIN-76A diet since parturition, and treated with DMBA on day 50. At day 100 postpartum (shortly after the first tumors can be palpated), we switched one group to 250 mg genistein/kg AIN-76A diet. The results showed no significant difference in tumor formation or adenocarcinoma development. We concluded that 1) rats not exposed to genistein prepubertally do not receive protection from genistein after tumors have developed, 2) genistein does not promote existing mammary tumors, and 3) that genistein exposure must occur prepubertally to exert a chemopreventive effect.

In the second tumorigenesis experiment, we investigated the potential of a combination of prepubertal and adult genistein exposure to protect against DMBA-induced mammary cancer. The purpose of this experiment was to determine if early critical exposure (prepubertal) to genistein would influence how the adult animal would respond to future genistein treatment. Group 1 was fed AIN-76A diet containing 250 mg genistein/kg diet, starting from parturition through day 21 only, and then AIN-76A onward (Gen/DMBA/Zero). Group 2 was fed the genistein diet from parturition through day 21, then AIN-76A only through day 100 postpartum and then from day 100, the genistein-containing diet (Gen/DMBA/Gen). Group 3 consisted of rats exposed to AIN-76A diet throughout the study (Zero/DMBA/Zero). All animals received 80 mg DMBA/kg BW at day 50. As seen in Figure 4 of reference 8 (enclosed publication), prepubertal genistein only exposure (Gen/DMBA/Zero) provided protection against DMBA induced mammary cancer (Zero/DMBA/Zero), confirming our previous work (1, 2). Furthermore, genistein fed to adult rats already exposed to genistein prepubertally (Gen/DMBA/Gen) resulted in an added level of protection compared those exposed to genistein prepubertally only (Gen/DMBA/Zero).

Please note that Figure 4 of reference 8 (enclosed publication) contains final data with more animals (25 rats/group) compared to the preliminary data presented in our 01 DOD report.

Specific Aim 2) To investigate genistein imprinting by methylation of estrogen receptor (ER) genes as the mechanism for mammary cancer prevention.

This aim has proven to be more difficult. First, Dr. Wang, who was working on this project, assumed new responsibilities and, 2) we have not been able to obtain the sequence of the ER-beta promoter. The personnel problem has be rectified. Dr. Nadejda Lopatina, who has specific experience with DNA methylation studies has been hired to replace Dr. Wang. A copy of her biographical sketch is enclosed. To date, she has demonstrated that DNA-methyltransferase activity using calf thymus DNA as substrate is increased in mammary glands of rats treated with genistein. In a preliminary experiment (in 2 samples only) measuring methyltransferase activity, with non-methylated oligonucleotide template as substrate, no change in enzyme activity (de novo methylation) was noted. On the other hand, using hemi-methylated DNA as substrate (a 60-mer oligonucleotide template containing CpG sequence), there was a 49% increase in DNA methylation. The latter assay is reflective of remethylation of already existing methylated DNA.

	Methyltransferase Activity (cpm/ ug protein)		
Treatment	Calf Thymus DNA	Non-methylated DNA	Hemi-methylated DNA
Control	725 ± 20	2303	2407
Genistein	974 ± 21 ($p<0.05$)	2320	3582

Should the increased methylation activity translate into increased methylation of the ER-alpha receptor, this would confirm our hypothesis that down-regulated ER-alpha protein occurs as a consequence of gene silencing, an imprinted mechanism to explain genistein chemoprevention.

KEY RESEARCH ACCOMPLISHMENTS

1) Dietary genistein given to adult female rats after tumors were initiated did not alter the multiplicity of mammary tumors. This can be interpreted as genistein not exerting a chemotherapeutic effect on existing tumors, and genistein not exacerbating development of previously existing mammary tumors.

2) On the other hand, dietary genistein to adult rats exposed prepubertally to genistein provides additional protection against mammary cancer. Prepubertal genistein exposure appears to "imprint for additional adult genistein chemoprevention.

3) Preliminary studies indicate that genistein treatment increases DNA methyltransferase activity (maintenance methylation), suggesting that DNA methylation and gene silencing may be molecular mechanism of genistein chemoprevention of mammary cancer.

REPORTABLE OUTCOMES

Publication

Lamartiniere, C.A., Cotroneo, M.S., Fritz, W.A., Wang, J. Roycelynn Mentor-Marcel, R.-M. and Ada Elgavish, A. Genistein Chemoprevention: Timing and Mechanisms of Action in Murine Mammary and Prostate. J. Nutrition. 132: 552S-558S, 2002.

Presentations

Fourth International Symposium on the Role of Soy in Preventing and Treating Chronic Disease: Dietary Genistein Protects Against Mammary and Prostate Cancers. San Diego, CA. Nov 4-7, 2001.

Roche Vitamins and Fine Chemicals. Dietary Genistein Protects Against Mammary and Prostate Cancers. Basel Switzerland. July 5, 2002.

Era of Hope Breast Cancer Meeting (DOD). Symposium Presenter: Genistein Programming Against Breast Cancer. Orlando FL. Sept. 25-28, 2002.

REQUEST FOR MODIFICATION

We have not been able to obtain the DNA sequence of the rat ER-beta promoter from Dr. J.-A. Gustaffson (Karolinska Institute, Sweden) as anticipated, nor does the Gene Bank have it. Accordingly, we request permission to measure DNA methylation of the ER-alpha gene promoter only.

Because of the delay in personnel appointment and in measuring DNA methylation of the ER-alpha promoter, we have requested and received a no cost extension by UAB and DOD to complete the work. The samples have been generated and are frozen, waiting to be processed.

CONCLUSIONS

We conclude that dietary genistein in adult life is only effective in protecting against chemically-induced mammary cancer if the female mammary gland has already been imprinted prepubertally. Our measurement of DNA methyltransferase activity suggest that genistein chemoprevention may be due to DNA methylation and gene silencing.

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APPENDICES

Biographical Sketch of Dr. Nadejda Lopatina

One publication

Lamartiniere, C.A., Cotroneo, M.S., Fritz, W.A., Wang, J. Roycelynn Mentor-Marcel, R.-M. and Ada Elgavish, A. Genistein Chemoprevention: Timing and Mechanisms of Action in Murine Mammary and Prostate. J. Nutrition. 132: 552S-558S, 2002.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Lopatina, Nadejda, Ph.D.	Postdoctoral Fellow Department of Biology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Moscow State Univ., Moscow, Russia	M.S.	1974	Biochem/Virology
Inst. of Biomed. Chem., Moscow, Russia	Ph.D.	1980	Biochemistry

POSITIONS

1974-1976 Research Associate; Institute of Biomedical Chemistry, Moscow, Russia
1976-1990 Research Scientist; Institute of Biomedical Chemistry, Moscow, Russia.
1990-1994 Senior Research Scientist: Institute of Biomedical Chemistry, Moscow, Russia
1994-1997 Visiting Scientist: National Center for Toxicological Research, Jefferson, AR, USA.
1997-1998 Research Associate: University of Medical Sciences Little Rock, AR , USA
1998-1999 Visiting Scientist; National Center of Toxicological Research, Jefferson, AR, USA
1999-present Postdoctoral Fellow; Biology and Dental School, UAB, Birmingham, AL, USA.

PROFESSIONAL SOCIETIES

Russian Biochemical Society
DNA Methylation Society
American Association for Cancer Research

SELECTED PUBLICATIONS (Relevant partial listing of 38 total):

Sharkova E.V., Lopatina N.G., Durisz A., Feldes I., Nikolskaya I.I: DNA-methylating system from nucleus from chicken liver in normal state and under viral transformation. Molekulyarnaya Genetika, Mikrobiologiya I Virusologiya 3, 13-17, 1993

Nikolskaya I.I., Scharkova E.V., Zenina E.N., Lopatina N.G., Atachanova B.A., Koneeva A.E., Kovalev L.I., Shishkin S.S: DNA-methylases from human thyroid formations - nodular and diffuse goiter. Biokhimiya 59, 1616-1624, 1993 (Russian)

Lopatina N.G., Nikolskaya I.I,. Kovalev L.I., Scharkova E.V., Erschova E.L., Kirkel A. Z. and Shishkin S. S.: DNA methylase activity in human myocardium is associated with actin proteins. Molecular Biology 29, 513-518, 1995.

Pogribny I., Basnakian A.G., Barbara J. Miller, Nadejda G. Lopatina, Lionel A. Poirier L. and S. Jill James: DNA strand breaks in genomic DNA and within the P53 gene are associated with hypomethylation in livers of folate/methyl deficient rats. Cancer Research, 55, 1894-1901, 1995.

N.G. Lopatina, B.F.Vanyushin, G.M. Cronin, and L.A. Poirier: Elevated expression and altered pattern of DNA methyltransferase activity in liver of rats fed a methyl-deficient diet. Carconogenesis 19, 1777-1781, 1998.

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Hammons G.J., Yan Y., Lopatina N.G., Jin B., Wise C., Blann E.B., Poirier L.A., Kadlubar F.F., Lyn-Cook B.D.: Increased expression of hepatic DNA methyltransferase in smokers. Cell Biol. Toxicology 15, 389-394,1999

N.G. Lopatina, C.A. Cooney, C.K. Wise and L.A. Poirier: DNA methyltransferase activity in actin from porcine heart. Biochim et Biophys. Acta, submitted.

Fourth International Symposium on the Role of Soy in Preventing and Treating Chronic Disease

Genistein Chemoprevention: Timing and Mechanisms of Action in Murine Mammary and Prostate[1,2]

Coral A. Lamartiniere,*†[3] Michelle S. Cotroneo,* Wayne A. Fritz,* Jun Wang,* Roycelynn Mentor-Marcel*** and Ada Elgavish†**

*Department of Pharmacology and Toxicology, †University of Alabama at Birmingham Comprehensive Cancer Center, and the **Department of Genomics and Pathology, University of Alabama at Birmingham, Birmingham, AL 35294*

ABSTRACT We investigated the potential of genistein, the primary isoflavone of soy, to protect against breast and prostate cancers in animal models. For mammary cancer studies, Sprague-Dawley rats were fed AIN-76A diet ± 250 mg genistein/kg diet. Dimethylbenz[a]anthracene was administered by gavage at d 50 postpartum to induce mammary tumors. Mammary cancer chemoprevention was demonstrated after prepubertal and combined prepubertal and adult genistein treatments but not after prenatal- or adult-only treatments, demonstrating that the timing of exposure to genistein is important for mammary cancer chemoprevention. The cellular mechanism of action was found to be mammary gland and cell differentiation, as shown by whole-mount analysis and β-casein expression. An imprinting effect was shown for epidermal growth factor receptor expression in mammary terminal end buds. For prostate cancer studies, we used two models. The first was a chemically (*N*-methylnitrosourea) induced prostate cancer rat model. Genistein in the diet inhibited the development of invasive adenocarcinomas in a dose-dependent manner. The second model was a transgenic mouse model that resulted in spontaneously developing adenocarcinoma tumor of the prostate. Genistein in the diet reduced the incidence of poorly differentiated prostatic adenocarcinomas in a dose-dependent manner and down-regulated androgen receptor, estrogen receptor-α, progesterone receptor, epidermal growth factor receptor, insulin-like growth factor-I, and extracellular signal-regulated kinase-1 but not estrogen receptor-β and transforming growth factor-α mRNA expressions. We conclude that dietary genistein protects against mammary and prostate cancers by regulating specific sex steroid receptors and growth factor signaling pathways. J. Nutr. 132: 552S–558S, 2002.

KEY WORDS: • *genistein* • *chemoprevention* • *mammary* • *prostate* • *cancer*

Cancer is usually treated at the time of diagnosis, and chemoprevention is not usually considered until adulthood. However, because perinatal exposure to hormones and xenobiotics influences breast and prostate development and cancer, we have hypothesized that exposure to hormonally active nutritional chemicals during early windows of development plays a key role for cancer causation and prevention in these tissues.

The most convincing evidence indicating that environmental agents and early periods of development predispose for breast cancer is radiation exposure. Women exposed as teenagers to ionizing radiation are more susceptible for breast cancer than those exposed as adults (1,2). Moreover, early pregnancy or early exposure to the hormones of pregnancy reduces the incidence of breast/mammary cancer in women and animal models (3–5). This demonstrates that the early period of a woman's life is crucial for predisposition to or for protection against breast cancer.

Asian women consuming a diet high in soy products have a low incidence of breast cancer (6,7), yet Asians who immigrate to the United States and adopt a Western diet lose this protection. Soy-based diets are high in phytochemicals and quantitative results indicate that isoflavone phytoestrogens are normal constituents of human urine from subjects consuming large amounts of soy products (tofu, soy flour, soy milk, tempeh, etc.) (8). Genistein is the predominant isoflavone phytoestrogen found in soy.

[1] Presented as part of the Fourth International Symposium on the Role of Soy in Preventing and Treating Chronic Disease held in San Diego, CA, November 4–7, 2001. This conference was supported by Central Soya Company; Monsanto; Protein Technologies International; SoyLife Nederland BV/Schouten USA SoyLife; United Soybean Board; Archer Daniels Midland Company; Cargill Soy Protein Products/Cargill Nutraceuticals; Illinois Soybean Association/Illinois Soybean Checkoff Board; Indiana Soybean Board; Cyvex Nutrition; Nichimo International, Inc.; Nutri Pharma Inc.; Revival Soy; Solbar Plant Extracts Ltd.; Soyatech Inc.; AOCS Press; Dr. Soy Nutrition; Eurofins Scientific/Product Safety Labs; and Optimum Nutrition. This publication was supported by (in alphabetical order) the Indiana Soybean Board, the Kentucky Soybean Board, the South Dakota Soybean Research and Promotion Council, Soyfoods Council, Cargill, and the United Soybean Board. Guest editors for this symposium were Stephen Barnes and Mark Messina.

[2] This research was supported by National Institutes of Health Grant R01 CA61742 and DOD Grants DAMD 17-98-1-8582 and DAMD 17-00-1-0118.

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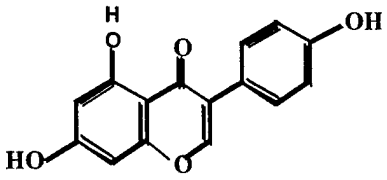

FIGURE 1 Structure of genistein.

Genistein is a planar molecule with an aromatic A-ring, has a second oxygen atom 11.5 Å from the one in the A ring, and has a molecular weight similar to those of the steroidal estrogens (**Fig. 1**). It has estrogenic properties in receptor binding assays (9,10), cell culture (11,12), and uterine weight assays (13–15). Genistein inhibits topoisomerase II (16), platelet-activating factor- and epidermal growth factor-induced expression of c-fos (17), diacylglycerol synthesis (18), and tyrosine kinases (19). It also inhibits microsomal lipid peroxidation (20) and angiogenesis (21). Genistein exhibits antioxidant properties (22–24) and was reported to induce differentiation of numerous cell types (25–27). Most of these mechanistic data were derived from in vitro studies.

Genistein and mammary cancer

To investigate the potential of perinatal genistein exposure to protect against chemically induced mammary cancer, female Sprague-Dawley rats were fed 0, 25 and 250 mg genistein/kg AIN-76A diet starting 2 wk before breeding (28). Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the University of Alabama at Birmingham Animal Care Committee. The dietary concentrations were chosen because they yield serum genistein concentrations in rats similar to blood genistein concentrations in men and women eating a diet high in soy (28,29). After parturition, dams and offspring were fed the same diets until time of weaning (d 21). From that time onward, all female offspring from the three treatment groups were fed AIN-76A diet only. At d 50 postpartum, dimethylbenz[a]anthracene (DMBA)[4] (80 mg/kg body) was administered by gavage to induce mammary tumors. Animals were palpated for tumors and necropsied at 180 d after DMBA treatment or when tumors developed to 2.5 cm in diameter. Control animals (zero genistein, DMBA) developed approximately nine tumors, whereas dietary genistein suppressed DMBA-induced mammary tumor development in a dose-dependent manner. Rats exposed to 25 and 250 mg genistein/kg AIN-76A diets had 7.1 and 4.3 mammary tumors, respectively (**Fig. 2**). This dietary genistein chemoprevention study is consistent with our previous work demonstrating that injections of pharmacologic doses of genistein during the neonatal and prepubertal periods suppressed chemically induced mammary tumor development (30,31). Recently, Hilakivi-Clarke et al. (32) confirmed that prepubertal exposure to genistein reduces mammary tumorigenesis.

In the next study, we addressed the possibility that genistein exposure, via the dam during the prenatal period only, might alter the female offspring's susceptibility for mammary cancer. One group of female rats was fed 250 mg genistein/kg AIN-76A diet during breeding and pregnancy. At parturition, the dams and offspring were switched to AIN-76A diet without genistein supplement. The control group for this experiment was made up of females fed AIN-76A diet from the time of breeding throughout the experiment. Dietary exposure to genistein, merely during the prenatal period, neither increased mammary carcinogenesis nor conferred protection against DMBA-induced (80 mg/kg body) mammary cancer (**Fig. 3**). These data confirm our previous prenatal genistein study using a lower dose of DMBA (40 mg/kg body) (29). Data from Figures 2 and 3 show that the critical window for genistein chemoprevention is the postnatal time of the perinatal period.

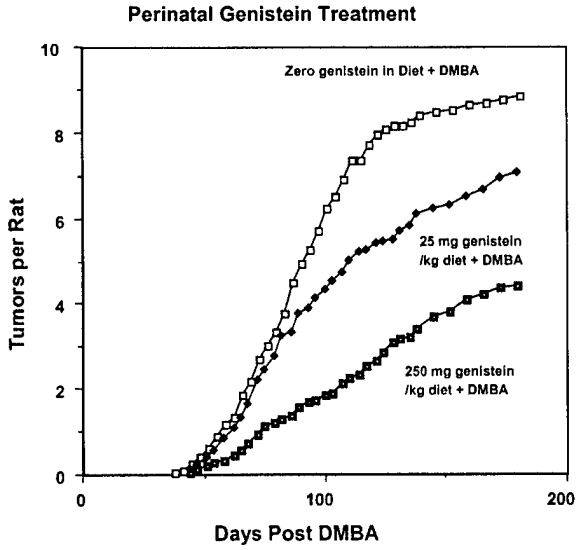

FIGURE 2 Ontogeny of palpable mammary tumors in female Sprague-Dawley CD rats exposed perinatally to genistein in the diet. Starting at time of breeding, dams were fed 0, 25 and 250 mg genistein/kg AIN-76A diet. After weaning, the offspring were fed AIN-76A diet only. On d 50 postpartum, female offspring were treated with 80 mg dimethylbenz[a]anthracene (DMBA)/kg body. [Modified from (28). Permission requested from Oxford University Press.]

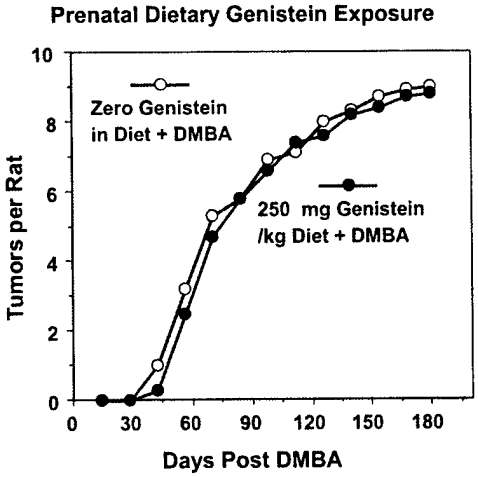

FIGURE 3 Ontogeny of palpable mammary tumors in female offspring of Sprague-Dawley CD rats fed genistein in the diet during pregnancy. Two groups of pregnant female rats (25 each) were fed 0 or 250 mg genistein/kg AIN-76A diet. At parturition both groups were fed AIN-76A diet until the time of necropsy (230 d postpartum). All offspring were treated with 80 mg DMBA/kg body on d 50 postpartum.

[4] Abbreviations used: DMBA, dimethylbenz[a]anthracene; EGF, epidermal growth factor; ER, estrogen receptor; MNU, methylnitrosourea; TRAMP, transgenic mouse prostate adenocarcinoma.

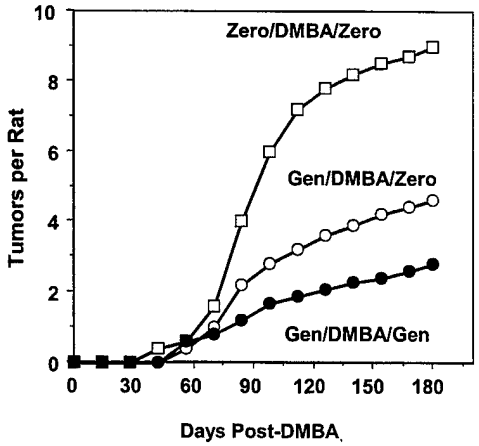

FIGURE 4 Adult dietary genistein effect on palpable mammary tumors in rats exposed prepubertally to genistein and as adults to DMBA. Group 1 was fed control AIN-76A diet starting from parturition and continued throughout the study (Zero/DMBA/Zero). Group 2 was fed AIN-76A diet containing 250 mg genistein/kg diet, starting from parturition through d 21 only and then AIN-76A onward (Gen/DMBA/Zero). Group 3 was fed genistein-containing diet from parturition through d 21, AIN-76A only through d 100 postpartum, and genistein-containing diet (Gen/DMBA/Gen) from d 100. All rats received 80 mg DMBA/kg body at d 50. Each group consisted of 25 rats.

Our data (Fig. 3) demonstrate that dietary genistein administered prenatally did not alter predisposition for mammary cancer. In contrast, Hilakivi-Clarke et al. (33) reported that injecting pregnant rats with genistein resulted in increased susceptibility of the offspring for mammary cancer. We speculated that this apparent contradiction might be due to different routes of administration and bioavailability in the two studies. Circulating genistein concentrations from 21-d fetal, 7-d neonatal and 21-d prepubertal rats exposed to 250 mg genistein/kg AIN-76A diet were determined to be 43, 726 and 1810 nmol/L, respectively (28,29). This demonstrates genistein bioavailability during postnatal life but poor bioavailability prenatally. Also, we determined that ~46% of circulating total genistein is free genistein 24 h after injection of rats (34). This is in contrast to <2% being free (aglycone) genistein from dietary administration (28). The bioavailability of injected genistein is substantially greater than that of oral genistein (23-fold). Hence, we conclude that route of administration and timing of exposure determines the metabolism, bioavailability and biological action of genistein.

Because breast cancer has been demonstrated to be estrogen-dependent, we have been concerned that genistein, a phytoestrogen, may contribute to mammary cancer development. More specifically, women who have been diagnosed with breast cancer inquire whether soy products, including genistein, will protect from or cause a recurrence of their cancer. We attempted to address this in a laboratory study. Rats were fed AIN-76A diet ± 250 mg genistein/kg diet at three periods, and all females were treated intragastrically with 80 mg DMBA/kg body. As seen in **Figure 4**, rats exposed to the control diet, AIN-76A only, from birth until the end of the experiment (Zero/DMBA/Zero) had the highest average number of tumors (9.0 tumors/rat). Rats exposed to genistein from d 1 to 21 postpartum only (Gen/DMBA/Zero) developed 4.5 tumors, which confirms our earlier work (28). Animals exposed to genistein from d 1 to 21 and 100 to 180 (Gen/DMBA/Gen) developed the fewest number of tumors (2.8 tumors/rat). The latter genistein feeding was initiated 50 d after the DMBA treatment, the time of onset of palpable mammary tumors. This demonstrates that genistein fed to adult rats previously exposed prepubertally to genistein provided these rats with additional protection against mammary cancer. Prepubertal genistein exposure seems to permanently affect the animal or mammary gland in a way that determines how that animal later responds to the same or similar chemical stimuli. In this case, genistein fed during the prepubertal period programmed future (adult) genistein response against mammary cancer susceptibility.

Table 1 summarizes the relationship among dietary genistein, timing of exposure and chemically induced mammary cancer in rats. Limiting exposure to dietary genistein to the prenatal or adult periods does not predispose or protect against mammary cancer. In contrast, exposure to dietary genistein during the prepubertal and prepubertal-plus-adult periods protected against chemically induced mammary cancer in rats. An epidemiological report using the Shanghai Cancer Registry, a case-control study, has shown an inverse relationship (50%) between adolescent (13–15 y old) soyfood intake and breast cancer incidence later in life (35).

Genistein mechanism of action in the mammary gland

Analysis of mammary gland morphology in rats treated with genistein revealed that its cellular mechanism of action is enhancement of mammary gland differentiation (28–31). We demonstrated that genistein administered to prepubertal rats reduced the number of terminal end buds and increased the number of lobules. Mammary terminal end buds are terminal ductal structures found primarily in young animals (and humans) and contain many undifferentiated epithelial cells (36,37). Terminal end buds are the most susceptible structures to chemical carcinogens; lobules are the terminal ductal structures most differentiated and least susceptible to chemical carcinogens.

Further evidence that genistein enhances differentiation was obtained by measuring β-casein in mammary glands. β-casein is a milk protein and biomarker of mature mammary glands and differentiated cells. Using Western blot analysis, we found that prepubertal genistein treatment increased β-casein expression in mammary glands of prepubertal and adult rats (**Fig. 5**). In the adult rats, β-casein was measured 30 d after genistein treatment.

One of the reasons cancer researchers have investigated

TABLE 1

Dietary genistein, timing of exposure and mammary cancer chemoprevention

Exposure period	Relative mammary tumor multiplicity[1]
No genistein	8.9
Prenatal genistein[2]	8.8
Adult genistein (after tumors)[3]	8.2
Prepubertal genistein[4]	4.3
Prepubertal and adult genistein[3,4]	2.8

Diets contained ± 250 mg genistein/kg AIN-76A.
[1] All rats were treated with 80 mg dimethylbenz[a]anthracene/kg body weight at d 50 postpartum.
[2] Prenatal treatment is throughout gestation.
[3] Adult treatment was initiated at 100 d postpartum.
[4] Prepubertal treatment was from d 1 to 21 postpartum.

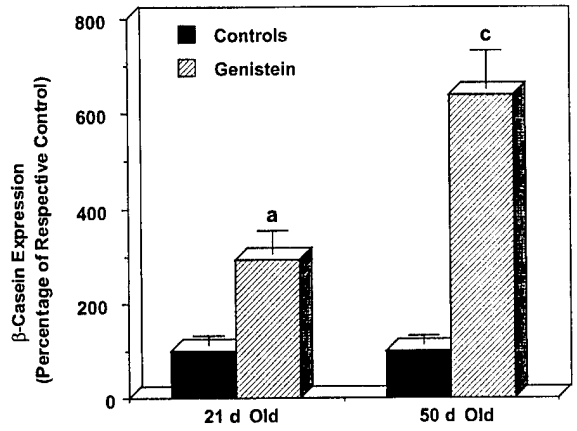

FIGURE 5 β-casein expression in mammary glands of rats treated prepubertally with genistein. Female Sprague-Dawley CD rats were injected subcutaneously with 500 μg genistein/g body or an equivalent volume of the vehicle, dimethylsulfoxide, on d 16, 18 and 20 postpartum. Western blot analysis was carried out to measure β-casein expression in mammary glands of 21- and 50-d-old rats (six animals per group). The β-casein antibody was a generous gift from Margaret Benton and Michael Gould of the University of Wisconsin, Madison.

genistein as a chemopreventive agent is the reports that it inhibits protein tyrosine kinases in vitro (18,19). As an extension of this, we investigated the potential of genistein to regulate the epidermal growth factor (EGF) receptor in vivo. In 21-d-old rats treated prepubertally with genistein, we found increased EGF receptor expression in mammary terminal end buds (38). Not only was this finding contrary to the in vitro reports, but this was surprising because we expected a chemopreventive agent to down-regulate the expression of this growth factor signaling pathway. However, when we extended our studies to 50-d-old rats treated prepubertally with genistein, we observed that EGF receptor expression was down-regulated in terminal end buds of these rats. We interpreted this to mean that early in postnatal life, genistein initially up-regulated the EGF-signaling pathway to enhance mammary gland development that resulted in early mammary gland differentiation. Reduced EGF signaling and decreased cell proliferation at 50 d when the DMBA was given was associated with reduced susceptibility to chemical carcinogenesis (28–31,36,37). Developmental modifications by a hormonally active chemical that result in altered biochemical or behavioral responses later in life was defined as imprinting (39–41). We speculate that down-regulated EGF receptor signaling in mammary terminal end buds at the time of carcinogen exposure plays a role in reduced mammary cancer development.

Genistein and mammary cancer chemoprevention summary

We have demonstrated that prepubertal and prepubertal-plus-adult genistein exposures protect against chemically induced mammary cancer in rats. We conclude that for genistein to protect against breast cancer, initial exposure must occur during the early sensitive period of mammary gland development, that is, the neonatal through prepubertal periods. The cellular mechanism of action of genistein is to enhance mammary cell differentiation (28–31). One identified biochemical mechanism is short-term and direct up-regulation of the EGF-signaling pathway that plays a role in cell differentiation (38). Paradoxically, this results in the epithelial cells of the mammary terminal end buds of adult animals having reduced EGF receptor expression. EGF signaling has been associated with cell proliferation; hence, we believe that down-regulated EGF receptor in adults contributes to the genistein chemoprevention. In reference to genistein in adults conferring additional protection when given to rats previously exposed to genistein compared with genistein-naive animals, we speculate that the early developmental effects have altered the molecular blueprint from which mammary cells respond to cancer initiators and promoters. Our laboratory data are consistent with the epidemiological report showing an inverse relationship between adolescent soyfood intake and breast cancer incidence later in life (35). We conclude that the most sensitive period for mammary cancer chemoprevention in the rat is the prepubertal period and in the human is probably the adolescent period.

Genistein and prostate cancer

Prostate cancer is the second leading cause of cancer death in men. Epidemiological data indicate that the incidence and mortality of prostate cancer are considerably lower in Asian populations than in U. S. and European populations (42), yet the incidence of precancerous lesions is the same for these populations (43). Upon emigration to the United States, Asian men have a greater risk for developing prostate cancer, and the earlier in life their arrival, the more closely their risk approaches that of American men (44).

One of the major differences between Asian and Western populations is diet. Asians have traditionally consumed a soy-based diet containing isoflavones, resulting in higher genistein concentrations in the blood and urine than those of American men (45,46). Our goal was to investigate the potential of genistein in the diet to protect against prostate cancer.

For the first prostate chemoprevention study, Lobund-Wistar rats were exposed to 0, 25 and 250 mg genistein/kg AIN-76A diet starting at conception and continuing until necropsy at age 11 mo (47). From d 50 to 66 postpartum, male offspring were given 33 mg flutamide/kg body daily by gavage to cause chemical castration. On d 67, 68 and 69, they were injected daily with 25 mg testosterone/kg body to stimulate mitosis. On d 70, all rats were anesthetized and 42 mg *N*-methylnitrosourea (MNU)/kg body was injected into the dorsal prostate for cancer initiation. One week after MNU administration, silastic implants of 25 mg testosterone were administered (and replaced every 12 wk) to stimulate mitosis and promote tumor growth. By age 40 wk, palpable prostate tumors were detectable. Rats were necropsied when 48 wk old or when they became moribund. In rats with small tumors, the tumors were confined to the site of MNU injection, demonstrating target organ specificity.

Rats fed the control diet, AIN-76A, and subjected to the carcinogenesis protocol developed 86.4% incidence of prostate tumors by 11 mo old (**Fig. 6**). Rats exposed to 25 and 250 mg genistein/kg diet had tumor incidences of 77.8% and 63.0%, respectively. The percentage of prostate tumors that were classified as invasive adenocarcinomas in rats fed 0, 25 and 250 mg genistein/kg diet were 77.3%, 61.1% and 44.4%, respectively. This was a dose-dependent significant decrease in prostate adenocarcinoma development (47). We conclude that lifetime dietary genistein protected against chemically induced prostate cancer development in rats.

The second model used for investigating genistein chemoprevention of prostate cancer was a transgenic mouse model that spontaneously develops prostate cancer, transgenic mouse

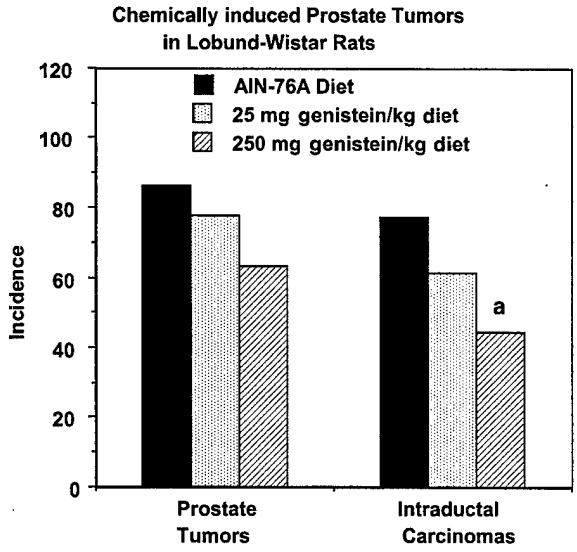

FIGURE 6 Prostate cancer incidence in Lobund-Wistar rats fed genistein in the diet. Lobund-Wistar rats were provided 0, 25 and 250 mg genistein/kg AIN-76A diet starting at conception. Male offspring were treated with 33 mg flutamide/kg body by gavage on d 50–66 and injected with 25 mg testosterone/kg on d 67–69; 42 mg methylnitrosourea/kg injected into the dorsolateral prostate on d 70; and 25-mg testosterone implants were started on d 77 (and replaced every 12 wk). Animals were necropsied when 48 wk old or when moribund. $^{a}P = 0.04$ compared with the AIN-76A diet group (Fisher exact test), and $P = 0.03$ by Cochran-Armitage trend test for tumor invasive adenocarcinomas. [Data modified from (47). Permission granted from Elsevier Science Ireland.]

prostate adenocarcinoma (TRAMP) (48). The TRAMP mouse was developed by using the prostate-specific probasin promoter to drive expression of the simian virus 40 early gene in the prostatic epithelium. The SV40 T antigen (Tag) acts as an oncoprotein through interactions with the p53 and retinoblastoma tumor-suppressor gene products. All TRAMP mice develop changes resembling human prostate intraepithelial neoplasia and poorly differentiated tumors, ultimately developing prostatic adenocarcinomas that metastasize to distant sites, primarily the lymph nodes, bone and lungs (49,50).

In our experiments (51), approximately one-half of the transgenic male mice displayed well-differentiated prostatic adenocarcinoma by 28 wk old; the other one-half was divided between moderately differentiated and poorly differentiated adenocarcinomas. To test the potential of genistein to prevent poorly differentiated adenocarcinomas, transgenic males were fed 0, 100, 250 or 500 mg genistein/kg AIN-76A diet, starting at 5–6 wk old. Mice remained on the diet until they were 28–30 wk old. The proportion of transgenic males that developed poorly differentiated adenocarcinoma was significantly reduced in a dose-dependent manner by dietary genistein (**Fig. 7**).

At necropsy, serum genistein concentration was determined and selected organs were weighed and prepared for histopathological evaluation (51). Serum genistein concentrations in mice on diets containing 0, 250 or 500 mg genistein/kg AIN-76A were 52 ± 33, 139 ± 70 and 397 ± 105 nmol/L, respectively, comparable with those found in Asian men on a regular soy diet (276 nmol/L) (45). As indicated by body and organ weights, dietary genistein had no toxic effect on TRAMP mice.

Sex steroid and growth factor signaling in the prostate

The interaction of sex steroid and growth factor signaling pathways is believed to be critical in the process of development and differentiation of hormone-responsive tissues and for cancer in the prostate (52). However, whether steroid hormones mediate the effects of growth factors or vice versa is unclear. Sex steroid-induced epithelial cell proliferation and differentiation have been associated with the coordinated induction of several peptide growth factors and their receptors, including some that are tyrosine kinase-dependent.

Nontransgenic and TRAMP mice were fed AIN-76A diet until 6 wk old, when one group of 14 TRAMP mice was fed 250 mg genistein/kg diet. An equal number of TRAMP and nontransgenic mice were fed AIN-76A diet only. At 12 wk old, the three groups of mice were killed and the dorsolateral prostates were collected. This is the period of prostate intraepithelial neoplasia and preneoplastic development in the prostate of TRAMP mice but before development of adenocarcinoma tumor (48,53,54). RNA was isolated and reverse-transcribed and the cDNA was amplified by polymerase chain reaction. Relative quantitative differences in cDNA were determined from data obtained during the exponential phase of amplification. In comparing prostates of transgenic and nontransgenic mice, we observed that androgen receptor, estrogen receptors (ER-α and -β), progesterone receptor, EGF receptor, transforming growth factor-α, insulin-like growth factor I and extracellular regulating kinase-1 mRNA transcripts were significantly higher in the transgenic mice (C. A. Lamartiniere and J. Wang, unpublished data, 2001). We speculate that increased sex steroid and growth factor signaling contribute to the increased incidence of spontaneously developing prostate cancer in transgenic mice.

In contrast, the prostates of transgenic mice fed the genistein-containing diet had reduced androgen receptor, ER-α, progesterone receptor, EGF-receptor, insulin-like growth factor I and extracellular regulating kinase-1 mRNA transcripts compared with prostates from TRAMP mice fed a

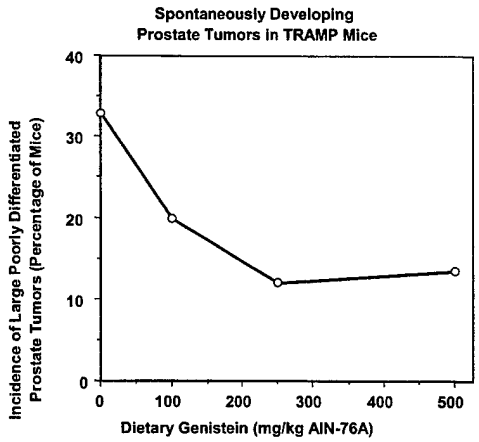

FIGURE 7 Genistein reduces the incidence of mice with advanced prostate tumors. The urogenital tract collected at necropsy was prepared for pathological evaluation of the prostate by established criteria (49,50). The results are the percentage of mice in each group with prostates displaying poorly differentiated adenocarcinomas; χ^2 test revealed that the frequency of transgenic mouse prostate adenocarcinoma (TRAMP) mice with poorly differentiated adenocarcinomas decreased significantly as a function of genistein in the diet ($P = 0.041$). [Data from (51). Permission granted from American Association for Cancer Research.]

diet devoid of genistein (C. A. Lamartiniere and J. Wang, unpublished data, 2001). ER-β and transforming growth factor-α mRNA were not altered by genistein. We speculate that genistein down-regulates expression of specific proteins and regulates cell proliferation and prostate cancer development. Should this down-regulation be extended to these sex steroid receptor and growth factor ligand and receptor proteins, this could provide a biochemical mechanism for the suppression of prostate cancer by genistein. Most interesting is that ER-β was not modulated by genistein. Not only does genistein bind with a greater affinity to ER-β than to ER-α (55), but the two ER have been shown to signal in different ways depending on ligand and response element. Also, ER-β is more involved in cell differentiation and ER-α is more involved in cell proliferation (56). Selective actions by genistein could explain both prostate gland differentiation via ER-β activation and reduced cell proliferation via down-regulated ER-α expression.

Genistein and prostate cancer summary

We demonstrated, in two animal models, that dietary physiological amounts of genistein can protect against chemically induced and spontaneously developing prostate cancers (47,51). We presented evidence that dietary genistein regulates, with specificity, sex steroid receptor and growth factor ligand and receptor mRNA expression. We speculate that these gene products contribute to chemoprevention of prostate cancer by genistein. Because postpubertal genistein exposure protects against prostate cancer development and can regulate sex steroid and growth factor signaling in animal models, we believe that genistein (or soy) can protect against prostate cancer in men.

DISCUSSION

We have demonstrated that the primary isoflavone component of soy, genistein, can protect against mammary (28–31) and prostate (47,51) cancers in rodent models. For mammary cancer protection, genistein exposure must first occur early in postnatal life. The importance of early mammary gland differentiation and carcinogenesis probably lies in pubertal development and estrogen surge, leading to oxidative DNA damage and cancer initiation. If the epithelial cells of the terminal end buds are differentiated, they are less susceptible for cancer (36,37). Also, we have demonstrated that early exposure to genistein exerts an imprinting-like effect on EGF receptor expression (38), a signaling pathway that plays a significant role in cell proliferation and cancer ontogeny. Imprinting is considered to set the pattern of gene expression early in development from which the adult responds; the pattern includes regulation of steroid receptor mechanisms and signal transduction. Consistent with this is the present demonstration that offspring imprinted early in life gain additional breast cancer protection with adult dietary exposure. Furthermore, we speculate that mammary cancer chemoprevention via cell differentiation and imprinting is not restricted to genistein. We believe that women imprinted by other means, for example, pregnancy or other nutritional differentiating chemicals, could benefit from ingesting soy as adults.

In reference to prostate cancer, we demonstrated that dietary genistein initiated at puberty suppressed spontaneously developing prostate cancer in transgenic mice (51). Short-term feeding of genistein from the pubertal to young adult period was able to down-regulate specific sex steroid receptor and growth factor ligand and receptor mRNA expression. We demonstrated that it is not necessary to give pharmacologic concentrations of genistein to get beneficial effects. TRAMP mice fed genistein had serum genistein concentrations (125–400 nmol/L) (51) comparable with blood genistein concentrations of Asians eating a traditional diet high in soy (276 nmol/L) (45,46). This supports our earlier report that dietary physiological amounts of genistein could regulate biochemical actions in the prostate, that is, EGF receptor expression in rats (57).

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